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Adoptive Immunotherapy to Increase the Level of Donor Hematopoietic Chimerism after Nonmyeloablative Marrow Transplantation for Severe Canine Hereditary Hemolytic Anemia

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ABSTRACT

Severe hemolytic anemia in Basenji dogs secondary to pyruvate kinase deficiency can be corrected by allogeneic hematopoietic cell transplantation (HCT) from littermates with normal hematopoiesis after conventional myeloablative or nonmyeloablative conditioning regimens. If the levels of donor chimerism were low (<20%) after nonmyeloablative HCT, there was only partial correction of the hemolytic anemia. We next addressed whether allogeneic cell therapy after nonmyeloablative HCT would convert mixed to full hematopoietic chimerism, achieve sustained remission from hemolysis, and prevent progression of marrow fibrosis and liver cirrhosis. Three pyruvate kinase–deficient dogs were given HCT from their respective dog leukocyte antigen–identical littermates after nonmyeloablative conditioning with 200 cGy of total body irradiation. Postgrafting immunosuppression consisted of mycophenolate mofetil and cyclosporine. All 3 dogs engrafted and had mixed hematopoietic chimerism with donor levels ranging from 12% to 55% in bone marrow. In 2 of the 3 dogs, there were decreases in the levels of donor chimerism so that at 25 weeks after nonmyeloablative HCT, hemolysis recurred that was associated with increased reticulocyte counts. All 3 dogs then had 2 serial infusions of donor lymphocytes (DLI) from their respective donors at least 20 weeks apart to convert from mixed to full donor chimerism. Both dogs with recurrence of hemolytic anemia after nonmyeloablative HCT achieved higher levels of donor chimerism, with donor contributions ranging from 47% to 62% in the bone marrow and 50% to 69% and 16% to 25% in the granulocyte and mononuclear cell fractions of the peripheral blood, respectively, and with remission of the hemolytic anemia. One dog responded after the first DLI, and 5 weeks after the second DLI, the other dog converted to full donor chimerism. At last follow-up, all these dogs showed clinical improvement, as determined by increasing hematocrits and normal reticulocyte counts. Analysis of the marrow 3 years after HCT showed normal cellularity, a normal myeloid-erythroid ratio, and no or minimal marrow fibrosis. Liver biopsies demonstrated normal histologies with no or minimal fibrosis. We conclude that DLI after nonmyeloablative HCT can increase the levels of donor cells contributing to hematopoiesis in recipients, inducing remissions of the hemolytic process and preventing complications associated with iron overload.

KEY WORDS

Nonmyeloablative transplantation • Hemolytic anemia • Donor lymphocyte infusions

INTRODUCTION

Pyruvate kinase (PK) deficiency in dogs results in a severe hemolytic anemia, which in turn results in a shortened life span because of both progressive myelofibrosis and liver cirrhosis [1,2]. Marrow transplantation of dogs with PK deficiency from hematologically normal, dog leukocyte antigen (DLA)–identical littermates after conventional high-dose conditioning corrected the hemolytic anemia, prevented the development of liver cirrhosis, and reversed the myelofibrosis and iron overload [3,4]. These observations—that the symptoms and sequelae of iron overload and

liver cirrhosis associated with severe hemolytic anemia could be ameliorated by conventional allogeneic marrow transplantation—set the stage for the first successful transplantation of allogeneic marrow in a human patient with thalassemia major [5]. Subsequently, this regimen was used for patients with other red blood cell disorders, such as sickle cell disease [6,7]. However, the risks of transplant-related complications, including those related to the myeloablative conditioning regimen, have made physicians hesitant to use this approach widely in patients with red blood cell disorders.

Recently, we reported that mixed hematopoietic chimerism established after nonmyeloablative conditioning and marrow transplantation was effective in correcting or delaying the development of myelofibrosis and liver cirrhosis in dogs with PK deficiency [8]. The clinical responses correlated with the degrees of donor hematopoietic chimerism. A low degree of donor chimerism was not sufficient to prevent continued hemolysis. We previously reported that mixed hematopoietic chimerism in dogs not affected with PK deficiency remained stable even after infusions of donor lymphocytes (DLI) [9]. However, if DLI was administered from donors that were sensitized to recipient-specific minor histocompatibility antigens, full hematopoietic chimerism could be induced in recipients. It was hypothesized in that study that suppressor cells in recipients with mixed chimerism prevented primary allorecognition and subsequent sensitization of newly infused T cells from nonsensitized donors. However, T cells in the DLI from donors sensitized to recipient minor histocompatibility antigens bypassed or overwhelmed this suppressor cell mechanism responsible for maintaining the state of mixed hematopoietic chimerism, resulting in the conversion to full donor chimerism. Therefore, in the next phase of these studies, PK-deficient dogs with mixed hematopoietic chimerism after nonmyeloablative hematopoietic cell transplantation (HCT) were given DLI from donors that had been sensitized to the recipients to induce full donor hematopoietic chimerism.

MATERIALS AND METHODS

Experimental Animals

Three dogs, a Beagle/minimongrel and 2 Basenji/Lhasa Apsos (7-11 months old; 6-14 kg body weight) were raised either at the Scott-Ritchey Research Center, Auburn University (Auburn, AL; n = 2) or at the Fred Hutchinson Cancer Research Center (n = 1). None of the dogs with PK deficiency had received transfusions of blood products before HCT. They were dewormed and vaccinated for rabies, distemper, leptospirosis, hepatitis, and parvovirus. They were housed in an American Association for Accreditation of Laboratory Animal Care–accredited facility in standard indoor runs and were provided commercial dog chow and chlorinated tap water ad libitum. Animal holding areas were maintained at 21°C ± 1°C with 50% ± 10% relative humidity, with at least 15 air changes per hour of 100% conditioned fresh air. The dogs were on a 12-hour light/dark full-spectrum lighting cycle with no twilight. Research was conducted according to the principles outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences, National Research Council. The research protocol was approved by the Institutional Animal Care and Use Committee of the Fred Hutchinson Cancer Research Center.

PK Genotype Assessment

PK deficiency in dogs that results in hemolytic anemia is an autosomal recessive disorder. The mutant R-type PK gene results from a single nucleotide deletion (ΔC^{433}). The PK genotype for PK deficiency in dogs was confirmed by genetic testing as previously described [10,11].

Histocompatibility Assessment

Littermate donor-recipient pairs were chosen on the basis of identity by highly polymorphic major histocompatibility complex class I and II microsatellite markers [12]. In addition, specific DLA-DRB1 allelic identity was determined by direct sequencing [13].

Bone Marrow Transplantation and Supportive Care

Total Body Irradiation, Postirradiation Care, and Postgrafting Immunosuppression. Recipient dogs with PK deficiency were given 200 cGy of total body irradiation at 7 cGy/min from 2 opposing cobalt 60 sources (n = 2) or from a Clinac 4 linear accelerator (n = 1; JM Co., San Jose, CA) [8,14,15]. Donors were DLA-identical littermates not affected by PK deficiency. Donor marrow was infused intravenously at doses of 2.2 to 2.7 $\times$ 10^9 cells per kilogram (median, 2.4 $\times$ 10^9 cells per kilogram) within 4 hours of total body irradiation. The day of marrow grafting was designated as day 0. Recipients were given cyclosporine 15 mg/kg twice daily orally on days −1 to 35 and mycophenolate mofetil 10 mg/kg twice daily subcutaneously on days 0 to 27 after transplantation.

All 3 dogs were given standard postgrafting care that included oral nonabsorbable antibiotics 3 times daily, neomycin sulfate, and polymyxin sulfate combined with systemic enrofloxacin, which were administered from day −5 until the day of white blood cell (WBC) count recovery to >1000/μL. Red blood cell and platelet transfusions from unrelated donors were given clinically as indicated. All transfused blood products were irradiated with 2200 cGy from a cesium

137 source. The dogs' clinical status was assessed twice daily.

Hematology. Hematology examinations consisting of complete blood counts and WBC differentials were performed at least twice before HCT and for a minimum of 5 days per week subsequently until recovery of red blood cell, granulocyte, and platelet counts, and then they were generally performed once a week until 4 to 6 months after HCT. The samples were collected into a tube containing EDTA. Automated hematologic analyses of blood samples were performed on a Sysmex E 2500 (Baxter, Chicago, IL). Blood smears were stained with Wright-Giemsa for WBC differentials and with new methylene blue for manual reticulocyte counts.

Marrow Biopsy, Aspiration, and Liver Biopsy. Marrow for transplantation was aspirated from the donors under general anesthesia through long needles inserted into humeri and femora, and for bone marrow evaluations, dogs were sedated [8]. Marrow aspiration and biopsies were performed before HCT, 25 weeks after HCT, 20 weeks after the first sensitized DLI, 20 weeks after the second sensitized DLI, and at 3 years after HCT. Liver biopsies were performed under general anesthesia and ultrasound guidance.

Serum Chemistry. Serum samples from dogs were collected before HCT and thereafter at least once a week for the first month, once every other week for the second month, and then at 3- to 4-month intervals to measure concentrations of total bilirubin, direct bilirubin, lactic dehydrogenase (LDH), glutamic-pyruvic transaminase (SGPT), γ-glutamyl transpeptidase, and alkaline phosphatase. Erythrocyte PK activity was also measured before HCT, 25 weeks after transplantation, 20 weeks after sensitized DLI, 20 weeks after a second sensitized DLI, and 3 years after HCT.

Sensitization of the Stem Cell Donor and Collection of Lymphocytes for Infusion

Donors were sensitized against recipient minor histocompatibility antigens (mHA) by subcutaneous placement of two 2-cm skin grafts from the recipient dog onto the bilateral flanks [9]. The skin grafts were performed once a week for 4 consecutive weeks. Within 7 days after the fourth skin graft, the donors underwent Cobe-apheresis collection of mononuclear cells. The leukapheresis product from the sensitized donor was administered intravenously on the same day as the collection. Before infusion, the $CD3^+$ content of the leukapheresis product was determined by fluorescence-activated cell-sorter analysis (Becton Dickinson, San Jose, CA) by using anti-CD3 fluorescein-conjugated monoclonal antibody 17.6F9.

Engraftment and Chimerism Study

Marrow engraftment was assessed by sustained recoveries of granulocyte and platelet counts after the postirradiation nadirs, by histologic features of the marrow from biopsy, and by documentation of donor cells in the marrow (including red blood cell precursors) and among granulocytes and mononuclear cells from the peripheral blood by using polymorphic microsatellite markers in a polymerase chain reaction–based assay [16]. The microsatellite marker technique detected 2.5% to 97.5% mixtures of donor and host cells. Peripheral blood cells were separated by Ficoll-Hypaque density gradient. Red blood cell precursors were isolated by cell sorting (Vantage; Becton Dickinson) on CD45-negative/dim and CD71-positive cells, which represented cells at an intermediate stage of erythroid development [8,17]. Canine CD45 antibody (CA12.10C12) was obtained from P.F. Moore (University of California, Davis, CA). The CD71 antibody against the human transferrin receptor (DAKO, Carpinteria, CA) stains canine marrow cells.

Assessment of Treatment Effect

Correction of disease manifestations after transplantation was assessed by evaluating hematocrits, reticulocyte counts, LDH, bilirubin, marrow and liver iron stored, and marrow and liver histology [18,19]. Complete resolution of hemolysis was defined as normalization of reticulocyte counts (<2%) and of hematocrits (37%-51%) and normal myeloid-erythroid (M:E) ratios in the marrow. A partial response was considered when 2 criteria were met: a >50% increase in hematocrit and a >50% reduction in reticulocyte counts. Tissue iron concentrations were determined on fresh samples by a bichromatic end point with a DuPont Dimension Analyzer (DuPont, Wilmington, DE) [18]. Gomori trichrome stain was used for fibrosis evaluation. Marrow fibrosis was classified as grade 1 (25% fibrosis), 2 (50% fibrosis), 3 (75% fibrosis), and 4 (100% fibrosis) [19].

RESULTS

Clinical Status of Dogs before HCT

The baseline characteristics of the PK-deficient dogs are summarized in Table 1. All dogs had evidence of hemolysis, with markedly decreased hematocrits and increased reticulocyte counts. Serum LDH levels were increased from 868 to 1605 U/L (median, 1049 U/L), reflecting the presence of the hemolytic process. The liver enzymes and serum bilirubin levels were within the normal range. Haptoglobin levels were low. Erythrocyte PK activity was normal. Marrows were hypercellular with mild to moderate fibro-

Table 1. *Clinical Status of PK-Deficient Dogs before and after Nonmyeloablative HCT and DLI*

Dog No.	Age (mo)	Hematocrit* (%)	Reticulocytes† (%)	LDH‡ (U/L)	Bilirubin§ (mg/dL)	PK Activity‖ (U/g hemoglobin)	Iron in Marrow¶ (μg/g)	Degree of Marrow Fibrosis# (0-4)	Marrow M:E** Ratio	Liver Histology	Liver Iron (μg/g)††
Before transplantation											
E854	7	18.7	43.4	868	0.3	9.8	135	1	1:1	ND	ND
E919	11	21.4	16.0	1605	0.4	12.5	225	1	Not reported	ND	ND
E920	11	27.8	30.0	673	0.3	22.5	477	1	1:5	ND	ND
25 wk after nonmyeloablative transplantation and before the first DLI											
E854	13	18.2	9.9	ND	ND	37.9	882	1	2:1	ND	ND
E919	17	43.1	3.1	1028	0.1	4.9	166	1	3:2	ND	ND
E920	17	27.7	10.2	1451	0.2	9.6	1101	1	3:2	ND	ND
20 wk after the first sensitized DLI and before the second DLI											
E854	19	35.3	3.8	717	0.1	9.5	122	ND	ND	ND	ND
E919	22	45.6	0.7	380	0.1	5.5	241	ND	ND	ND	ND
E920	22	40.3	1.0	668	0.2	2.4	582	ND	ND	ND	ND
After the second sensitized DLI: last follow-up											
E854	45	41.4	0.4	126	0.1	8.4	318	0-1	5:1	Normal; grade 0 fibrosis	1826
E919	47	47.1	1.8	250	0.1	7.9	273	0	3:2	Normal; grade 0 fibrosis	877
E920	47	44.8	0.6	163	0.1	6.4	697	0-1	3:2	Normal; grade 0-1 fibrosis	1729

*Normal range for dogs, 37% to 51%.

†Normal range for dogs, 0% to 2.0%.

‡Normal range, 90 to 250 U/L.

§Normal range for dogs, 0 to 0.3 mg/dL.

‖Normal range, 9.0 to 22.0 U/g hemoglobin.

¶Normal range, 530 to 900 μg/g.

#Marrow fibrosis was classified as grade 0 (none), 1 (25% fibrosis), 2 (50% fibrosis), 3 (75% fibrosis), and 4 (100% fibrosis).

**Normal M:E ratio, 3:2 to 5:1.

††Normal values obtained from 6 control dogs were 700 to 1700 μg/g for liver.

DLI indicates donor lymphocyte infusion; HCT, hematopoietic cell transplantation; ND, not done; LDH, lactic dehydrogenase; PK, pyruvate kinase.

sis (grade 1). The M:E ratio was increased in all 3 dogs.

Nonmyeloablative HCT

Nonhematopoietic Toxicities and Graft-versus-Host Disease. Nonmyeloablative HCT was well tolerated. Two dogs showed mild increases in liver enzyme levels (SGPT and alkaline phosphatase) in the first 4 to 6 weeks after transplantation; this may have been related to cyclosporine. No other toxicities or significant changes in serum chemistries were seen. None of the dogs developed acute or chronic graft-versus-host disease (GVHD).

Hematopoietic Engraftment and Response after HCT. All 3 dogs showed sustained allogeneic engraftment after HCT (Figure 1). One dog (E919) had sustained mixed hematopoietic chimerism (>60% donor chimerism) in the granulocyte fraction of the peripheral blood (Figure 1B). The other 2 dogs (E854 and E920) achieved >50% donor chimerism early after transplantation, but this decreased to <5% before the first DLI (Figure 1A and C). The levels of donor chimerism in the peripheral blood mononuclear cell (PBMC) fraction were sustained in all 3 dogs at 5% to 30% (Figure 1A-C). Clinical improvement was noted initially in all 3 dogs, with hematocrits increased to 40% (normal range, 37%-51%) and reticulocyte counts decreased to 1% (the normal range for dogs is 0%-2%). However, the late decreases in myeloid chimerism in 2 dogs (E854 and E920) were associated with recur-

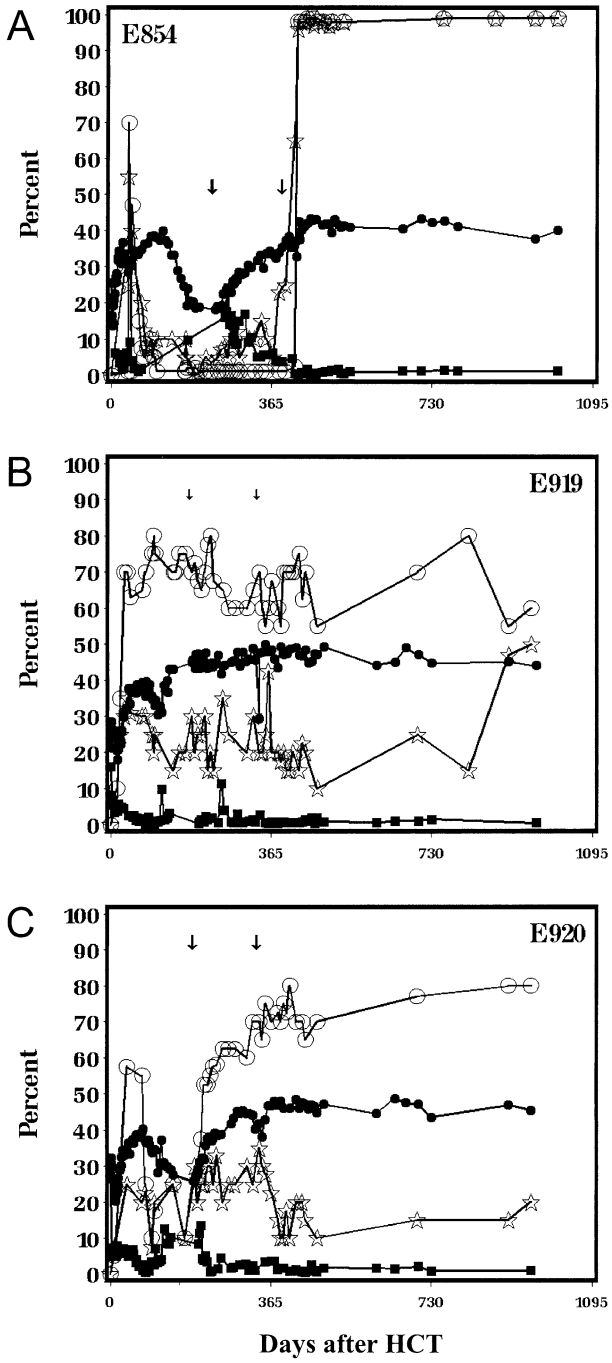

Figure 1. Donor chimerism levels in the granulocyte and mononuclear fractions of the peripheral blood, hematocrit, and reticulocyte counts across time after transplantation. Early after nonmyeloablative stem cell transplantation in 3 dogs with pyruvate kinase deficiency and hemolytic anemia, the levels of donor chimerism in the myeloid compartment (granulocyte fraction) decreased in 2 dogs (E854 and E920) with a recurrence of hemolytic anemia. All dogs had 2 infusions of donor lymphocytes (↓), and both dogs with the recurrence of hemolytic anemia had an increase in donor chimerism in the myeloid compartment and resolution of hemolytic anemia. In (A), (B), and (C), the hematocrit, reticulocyte count, and levels of donor chimerism in the peripheral blood mononuclear cell (PBMC) and granulocyte fractions are shown over time for dogs E854, E919, and E920, respectively. ● indicates hematocrit; ■, reticulocytes; ○, donor chimerism in the granulocyte fraction; ☆, donor chimerism in the PBMC fraction.

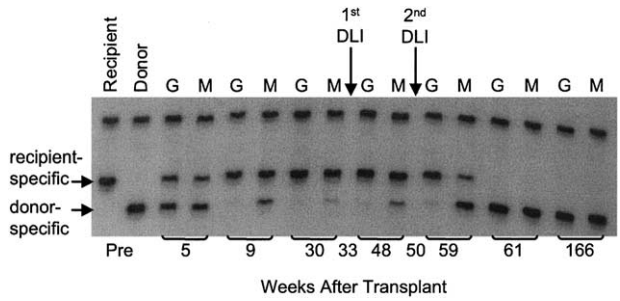

Figure 2. A representative sample of microsatellite marker studies of donor and recipient (E854) cells before transplantation and recipient cells after transplantation. After second donor lymphocyte infusion (DLI), there was a rapid conversion to complete donor chimerism (as noted in Figure 1A). G indicates peripheral blood granulocyte fraction; M, peripheral blood mononuclear cell fraction.

rence of the hemolytic anemia (evidenced by decreases in hematocrits and increases in the reticulocyte counts). Dogs were observed for a minimum of 25 weeks before the first mHA-sensitized DLI.

DLI: Effect on Chimerism Levels and Manifestations of Hemolysis

At the time of the first DLI, hemolysis associated with decreases in the donor chimerism of the granulocyte fraction to <5% had recurred in 2 of the 3 dogs (Table 1; Figure 2). Before DLI, donors were sensitized to recipient-specific mHA by grafting skin from the HCT recipient.

With the first infusion of lymphocytes from the respective sensitized donors, dogs E854, E919, and E920 received 2.14, 1.80, and 2.70 $\times$ 10^8 $CD3^+$cells per kilogram from their respective sensitized donor. At 18 to 22 weeks after the first DLI, 1 dog (E920) had a substantial increase in donor chimerism in the granulocyte fraction to >60% that was associated with an increase in the hematocrit to >40% and a decrease in the reticulocyte count. No substantial changes occurred in the level of donor chimerism in the granulocyte or PBMC fractions in the other 2 dogs.

Because of persistent mixed chimerism in all 3 dogs and the persistence of hemolysis in 1 dog (E854) with <5% chimerism, a second sensitized DLI was performed (Table 1). Donors were sensitized with serial skin grafts from the recipient for the second time. Dogs E854, E919, and E920 then received 1.28, 1.33, and 0.67 $\times$ 10^8 $CD3^+$ cells per kilogram, respectively. Dog E854 converted to full donor chimerism (98%) in the granulocyte fraction within 5 weeks after the second sensitized DLI (Figures 1A and 2). The other 2 dogs remained mixed chimeras, with donor contributions ranging from 47% to 62% in marrow, 50% to 69% in granulocyte fractions, and 16% to 25% in lymphocyte fractions until last follow-up. A population of red blood cell progenitors en-

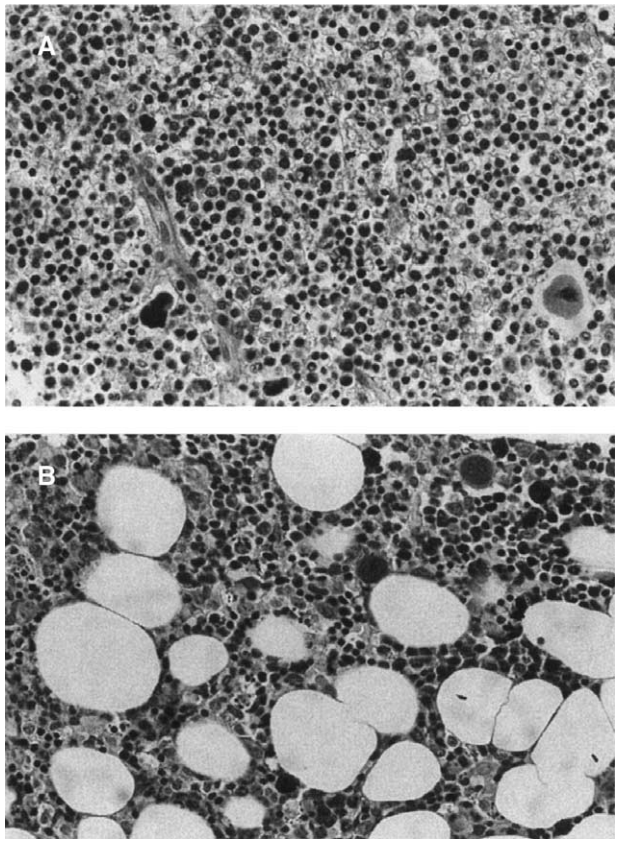

Figure 3. Marrow histology from a pyruvate kinase–deficient dog (E920) at baseline (A) and 3 years after nonmyeloablative transplantation and DLI (B). A, The marrow is hypercellular with a reversed M:E ratio. B, The marrow is normocellular with a normal M:E ratio and maturation of all 3 major cell lines. The vacuoles seen in B are fat cells that are observed in normocellular marrows from dogs (stain, periodic acid–Schiff; magnification, 300×).

riched for CD71 (transferrin receptor) was positively selected by flow cytometric cell sorting, DNA was extracted, and the percentage of donor chimerism was assessed. The level of donor chimerism was 99%, 50%, and 40% for E854, E919, and E920, respectively. These levels of chimerism were comparable to those noted in the granulocyte fraction of the peripheral blood for each of the dogs. All dogs showed clinical improvement, with increases in the hematocrit to 50% and sustained decreases in the reticulocyte counts to normal levels.

The clinical status of the dogs at 3 years after HCT and 2 years after the second DLI is described in Table 1. All 3 dogs had normal hematocrits and reticulocyte counts. Haptoglobin, LDH, and bilirubin levels were normal in all 3 dogs at last follow-up. Serum SGPT, γ-glutamyl transpeptidase, and alkaline phosphatase levels remained normal in general, although E854 had a mild increase in SGPT 1 year after HCT that persisted intermittently until the last follow-up (<2 times normal; E920 had an increased level after liver biopsy). Although PK activity was measured, these levels remained at normal levels and were not informative for the presence of recipient red blood cells affected by the inherited PK deficiency disorder. Three years after HCT, marrow cellularity and the M:E ratio in the marrow were normal (Figure 3). Minimal levels of marrow fibrosis persisted but had improved compared with the baseline evaluations. Marrow iron contents remained within normal levels. There were normal liver histologies in all dogs (Figure 4), with only minimal fibrosis noted in 1 dog. Liver iron content was mildly increased compared with controls.

DISCUSSION

Inherited red blood cell diseases, including PK deficiency, have been cured or stabilized by allogeneic marrow transplantation after conventional high-dose conditioning regimens [20]. The goal of marrow transplantation in these diseases has been the complete replacement of the defective cells with normal red blood cells. However, the toxicities from the myeloablative conditioning regimen required for the induction of complete donor hematopoietic chimerism have resulted in significant morbidity and mortality. More recently, it has been demonstrated that establishing mixed hematopoietic donor chimerism at substantial levels in patients with sickle cell disease or thalassemia may be sufficient for reducing transfusion requirements and complications from the disease [21,22]. We had previously shown in this canine model of hemolytic anemia secondary to PK deficiency that stable mixed chimerism could be induced after nonmyeloablative HCT, but if donor chimerism was less than 15% to 20%, resolution of the hemolysis

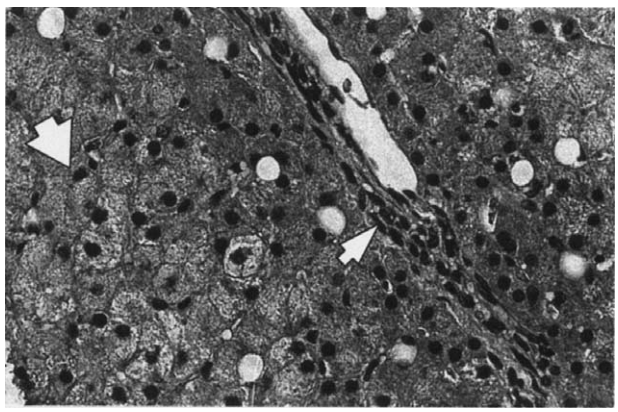

Figure 4. Normal liver histology in a pyruvate kinase–deficient dog (E920). Three years after nonmyeloablative transplantation, there were normal hepatocytes (large arrow) and bile ducts (small arrow). Iron deposition was normal. There was no development of significant liver disease from iron overload, which is normally associated with the natural history of pyruvate kinase deficiency in affected dogs.

was not achieved [8]. In this study, it was shown that DLI after nonmyeloablative HCT was capable of achieving complete or a high level of donor hematopoietic chimerism with complete resolution of the hemolytic anemia.

After nonmyeloablative HCT, all 3 dogs had a stable level of donor chimerism (5%-30%) in the PBMC fraction, but before DLI 1, 2 of the 3 dogs had had substantial decreases in donor chimerism in the myeloid compartment to <5%, with relapse of the hemolytic anemia. These decreases in donor chimerism after nonmyeloablative HCT without graft rejection suggested host resistance to the hematopoietic cell graft, possibly related to the hyperproliferative state of the recipient. After 2 infusions of recipient mHA-specific donor lymphocytes, 1 dog converted to complete donor chimerism (>98%), a second dog achieved a higher level of donor chimerism in the granulocyte fraction (>60%), and a third dog (already with substantial mixed hematopoietic chimerism) had no change in donor chimerism in either the granulocyte or PBMC fraction. All 3 dogs had sustained resolution of the hemolytic anemia, with increases of hematocrits to normal and decreases in the reticulocyte counts and serum LDH. Marrow histology became normal. Iron stores were normal or only mildly increased. In a disease process that would otherwise inevitably lead to liver cirrhosis, there was normal liver histology at 3 years after HCT. Erythrocyte PK activity was not informative in this study because the assay on the R-type isoenzymes was not performed. PK-deficient dogs have high in vitro erythrocyte PK activity from the M2-type PK isoenzyme, which is normally present in all tissues during fetal life and remains the major enzyme in erythroid precursors [10,23]. The R-type isoenzyme normally seen in mature red blood cells is absent, and the compensatory increase in the M2-type PK isoenzyme is not sufficient to prevent hemolysis. This is the first study in a large-animal model of an inherited red blood cell disease in which nonmyeloablative HCT followed by allogeneic cell therapy led to the establishment of effective levels of donor chimerism and a normal hematocrit without further evidence of significant hemolysis.

In studies of mice with inherited hemolytic disorders, normal red blood cells derived from donor hematopoietic stem cells in a mixed chimeric state may have an advantage contributing to the erythroid lineage compared with the genetically compromised cells. When large numbers of marrow cells from normal mice of the CBA strain ($CBA^{+/+}$) were injected into congenic PK-deficient mice with severe hemolytic anemia and splenomegaly without conditioning, the red blood cell numbers and PK activity of the mature red blood cells at 15 weeks were improved [24]. In other studies, normal donor cells transplanted in small numbers were sufficient to correct murine β-thalassemia [25,26]. Establishment of stable mixed chimerism after nonmyeloablative conditioning corrected the anemia in the murine model of sickle cell disease [27,28]. Engrafted mice with stable mixed chimerism demonstrated a higher level of donor chimerism in the red blood cell compartment than that measured in peripheral blood leukocytes. Partial correction of the anemia was observed in mice with levels of donor chimerism in the leukocytes of <2.5%, resulting in donor chimerism of 10% to 54% in the red blood cell compartment [27]. In another murine model of sickle cell disease, 70% normal myeloid chimerism was required to fully reverse the anemia. The higher levels of donor chimerism in peripheral red blood cells compared with leukocytes were likely related to the increased clearance of sickle red blood cells compared with normal mature red blood cells from the circulation. The observations in murine models of inherited red blood cell diseases after HCT are consistent with the observations made in the canine model of hemolytic anemia secondary to PK deficiency, in which higher donor chimerism levels in the leukocytes were associated with resolution of hemolysis and correction of the anemia. There was not an enrichment of the donor contribution to chimerism in red blood cell precursors. Although experience is still limited in the dog model, levels of donor chimerism less than 15% to 20% were associated with persistent hemolysis.

The presence of stable mixed hematopoietic chimerism after conventional conditioning and marrow transplantation from HLA-identical siblings has been associated with beneficial effects in patients with β-thalassemia and sickle cell disease. Patients with β-thalassemia who had leukocytes of donor origin as low as 20% had >80% to 95% normal β-globin chain synthesis [22,29]. The low percentages of donor leukocytes, as determined by microsatellite markers or fluorescent in situ hybridization analysis for the Y chromosome, were associated with much higher percentages of donor cells (fully differentiated) in the erythroid compartment. In a study of allogeneic HCT after conventional conditioning for sickle cell disease, 5 patients developed stable mixed chimerism [21]. In all 5 patients, there was a substantial reduction in hemoglobin S, and none of the patients experienced complications from sickle cell disease after the transplantation. Serial studies in 1 patient revealed the presence of 10% to 20% donor cells in the peripheral blood leukocytes and >70% hemoglobin A (which was stable for 3 years) in the red blood cells. However, donor chimerism of <5% would not be expected to be associated with resolution of the signs and symptoms associated with inherited red blood cell diseases. In this setting, therapy to increase the level of donor chimerism in the myeloid compartment may be of benefit. Although there was up to a 24% recurrence of

host cells in a report of 3 patients with thalassemia after myeloablative conditioning and allogeneic HCT, DLI resulted in conversion to complete donor chimerism [30].

Although there are potential benefits to the establishment of higher levels of donor chimerism with DLI after nonmyeloablative conditioning if symptoms of the inherited red blood cell disease persist, there are risks of GVHD and aplasia. GVHD may occur in as many as 60% of patients with relapsed CML after conventional HCT given DLI from nonsensitized donors [31,32]. To convert to complete donor hematopoietic chimerism in the dog model after nonmyeloablative conditioning and T cell–replete marrow transplantation, lymphocytes from the marrow donor that have been sensitized to recipient-specific mHA are required [9]. This is in contrast to converting mixed to full chimerism in dogs after myeloablative conditioning and T cell–depleted marrow transplantation. In the latter case, conversion to full hematopoietic chimerism did not require the use of lymphocytes from a sensitized donor [33]. In this study of nonmyeloablative HCT and DLI in the canine model of hemolytic anemia, none of the 3 dogs developed any complications secondary to DLI, and only 1 of the dogs achieved full hematopoietic chimerism. Because the dogs had to be bred for the genetic trait of PK deficiency from a limited number of affected dogs, there may have been less mHA disparity from which to generate an alloreaction. We previously reported on the experience of converting stable mixed to complete donor chimerism in 8 healthy dogs after nonmyeloablative transplantation with mHA-sensitized DLI [9]. All 8 recipients achieved conversion of donor chimerism to >98% within 2 to 12 weeks of the sensitized DLI. Complications from mHA-sensitized DLI were observed in 3 of the 8 recipients. Two dogs developed GVHD, of which only 1 required treatment. The third dog developed marrow aplasia and persistent pancytopenia. The incidence of complications after mHA-sensitized DLI in the dog model is comparable to that observed in humans from nonsensitized donors. A strategy to limit the risk of developing severe GVHD may include starting with low doses of $CD3^+$ T cells and then increasing in a stepwise fashion [34]. Future strategies to improve the safety of DLI may include the use of donor T cells genetically modified to express a suicide gene [35].

We have established proof of principle that the combination of nonmyeloablative HCT and delayed DLI for those recipients with low levels of donor hematopoietic chimerism is an effective strategy for the treatment of inherited red blood cell diseases. It is not proposed that clinical studies be conducted with DLI collected from donors sensitized to recipient antigens. This method was used to overcome the suppressor mechanism proposed to exist in recipients with mixed hematopoietic chimerism that suppressed the activity associated with DLI from a nonsensitized donor [9]. Other approaches for changing the levels of donor chimerism after DLI from nonsensitized donors that would be more feasible for future clinical studies are being investigated. In the clinic, efforts are still required to understand and overcome the problems of rejection and GVHD after nonmyeloablative HCT. For those patients with inherited red blood cell disorders, transfusion-induced sensitization may increase the risk of graft rejection. Further studies are needed to improve the safety of DLI for future clinical application, including better strategies for the management of GVHD.

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REFERENCES

1. Searcy GP, Tasker JB, Miller DR. Animal model of human disease: pyruvate kinase deficiency. *Am J Pathol.* 1979;94:689-692.
2. Prasse KW, Crouser D, Beutler E, Walker M, Schall WD. Pyruvate kinase deficiency anemia with terminal myelofibrosis and osteosclerosis in a beagle. *J Am Vet Med Assoc.* 1975;166:1170-1175.
3. Weiden PL, Hackman RC, Deeg HJ, Graham TC, Thomas ED, Storb R. Long-term survival and reversal of iron overload after marrow transplantation in dogs with congenital hemolytic anemia. *Blood.* 1981;57:66-70.
4. Weiden PL, Storb R, Graham TC, Schroeder ML. Severe hereditary haemolytic anaemia in dogs treated by marrow transplantation. *Br J Haematol.* 1976;33:357-362.
5. Thomas ED, Buckner CD, Sanders JE, et al. Marrow transplantation for thalassaemia. *Lancet.* 1982;2:227-229.
6. Walters MC. Hematopoietic cell transplantation for sickle cell disease, In: Thomas ED, Blume KG, Forman SJ. eds. *Hematopoietic Cell Transplantation.* Second Edition. Boston: Blackwell Science; 1999, 1145-1153
7. Lucarelli G, Clift RA. Marrow transplantation in thalassemia, In: Thomas ED, Blume KG, Forman SJ. eds. *Hematopoietic Cell Transplantation.* Second Edition. Boston: Blackwell Science; 1999, 1137-1144
8. Zaucha JM, Yu C, Lothrop CD Jr, et al. Severe canine hered-

itary hemolytic anemia treated by nonmyeloablative marrow transplantation. *Biol Blood Marrow Transplant.* 2001;7:14-24.
9. Georges GE, Storb R, Thompson JD, et al. Adoptive immunotherapy in canine mixed chimeras after nonmyeloablative hematopoietic cell transplantation. *Blood.* 2000;95:3262-3269.
10. Whitney KM, Goodman SA, Bailey EM, Lothrop CD Jr. The molecular basis of canine pyruvate kinase deficiency. *Exp Hematol.* 1994;22:866-874.
11. Whitney KM, Lothrop CD Jr. Genetic test for pyruvate kinase deficiency of Basenjis. *J Am Vet Med Assoc.* 1995;207:918-921.
12. Wagner JL, Burnett RC, DeRose SA, Francisco LV, Storb R, Ostrander EA. Histocompatibility testing of dog families with highly polymorphic microsatellite markers. *Transplantation.* 1996;62:876-877.
13. Wagner JL, Burnett RC, Works JD, Storb R. Molecular analysis of DLA-DRBB1 polymorphism. *Tissue Antigens.* 1996;48: 554-561.
14. Storb R, Yu C, Wagner JL, et al. Stable mixed hematopoietic chimerism in DLA-identical littermate dogs given sublethal total body irradiation before and pharmacological immunosuppression after marrow transplantation. *Blood.* 1997;89:3048-3054.
15. Yunusov MY, Georges GE, Storb R, et al. FLT3 ligand promotes engraftment of allogeneic hematopoietic stem cells without significant graft-versus-host disease. *Transplantation.* 2003; 75:933-940.
16. Yu C, Ostrander E, Bryant E, Burnett R, Storb R. Use of $(CA)_n$ polymorphisms to determine the origin of blood cells after allogeneic canine marrow grafting. *Transplantation.* 1994;58: 701-706.
17. Loken MR, Shah VO, Dattilio KL, Civin CI. Flow cytometric analysis of human bone marrow: I. Normal erythroid development. *Blood.* 1987;69:255-263.
18. Strasser SI, Kowdley KV, Sale GE, McDonald GB. Iron overload in bone marrow transplant recipients. *Bone Marrow Transplant.* 1998;22:167-173.
19. Dekmezian R, Kantarjian HM, Keating MJ, Talpaz M, McCredie KB, Freireich EJ. The relevance of reticulin stain-measured fibrosis at diagnosis in chronic myelogenous leukemia. *Cancer.* 1987;59:1739-1743.
20. Tanphaichitr VS, Suvatte V, Issaragrisil S, et al. Successful bone marrow transplantation in a child with red blood cell pyruvate kinase deficiency. *Bone Marrow Transplant.* 2000;26: 689-690.
21. Walters MC, Patience M, Leisenring W, et al. Stable mixed hematopoietic chimerism after bone marrow transplantation for sickle cell anemia. *Biol Blood Marrow Transplant.* 2001;7:665-673.
22. Kapelushnik J, Or R, Filon D, et al. Analysis of β-globin mutations shows stable mixed chimerism in patients with thalassemia after bone marrow transplantation. *Blood.* 1995;86: 3241-3246.
23. Black JA, Rittenberg MB, Standerfer RJ, Peterson JS. Hereditary persistence of fetal erythrocyte pyruvate kinase in the Basenji dog, In: Brewer GJ. ed. *The Red Cell.* New York: Alan R. Liss; 1978, 275-290
24. Morimoto M, Kanno H, Asai H, et al. Pyruvate kinase deficiency of mice associated with nonspherocytic hemolytic anemia and cure of the anemia by marrow transplantation without host irradiation. *Blood.* 1995;86:4323-4330.
25. Wagemaker G, Visser TP, van Bekkum DW. Cure of murine thalassemia by bone marrow transplantation without eradication of endogenous stem cells. *Transplantation.* 1986;42:248-251.
26. van den Bos C, Kieboom D, Wagemaker G. Correction of murine beta-thalassemia by partial bone marrow chimerism: selective advantage of normal erythropoiesis. *Bone Marrow Transplant.* 1993;12:9-13.
27. Kean LS, Durham MM, Adams AB, et al. A cure for murine sickle cell disease through stable mixed chimerism and tolerance induction after nonmyeloablative conditioning and major histocompatibility complex-mismatched bone marrow transplantation. *Blood.* 2002;99:1840-1849.
28. Iannone R, Luznik L, Engstrom LW, et al. Effects of mixed hematopoietic chimerism in a mouse model of bone marrow transplantation for sickle cell anemia. *Blood.* 2001;97:3960-3965.
29. Andreani M, Manna M, Lucarelli G, et al. Persistence of mixed chimerism in patients transplanted for the treatment of thalassemia. *Blood.* 1996;87:3494-3499.
30. Kapelushnik J, Or R, Aker M, et al. Allogeneic cell therapy of severe beta thalassemia major by displacement of host stem cells in mixed chimera by donor blood lymphocytes. *Bone Marrow Transplant.* 1997;19(suppl 2):96-98.
31. Collins RH Jr, Shpilberg O, Drobyski WR, et al. Donor leukocyte infusions in 140 patients with relapsed malignancy after allogeneic bone marrow transplantation. *J Clin Oncol.* 1997;15: 433-444.
32. Kolb HJ, Schattenberg A, Goldman JM, et al. Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. European Group for Blood and Marrow Transplantation Working Party Chronic Leukemia. *Blood.* 1995;86:2041-2050.
33. Kolb HJ, Gunther W, Schumm M, Holler E, Wilmanns W, Thierfelder S. Adoptive immunotherapy in canine chimeras. *Transplantation.* 1997;63:430-436.
34. Mackinnon S, Papadopoulos EB, Carabasi MH, et al. Adoptive immunotherapy evaluating escalating doses of donor leukocytes for relapse of chronic myeloid leukemia after bone marrow transplantation: separation of graft-versus-leukemia responses from graft-versus-host disease. *Blood.* 1995;86:1261-1268.
35. Bonini C, Ferrari G, Verzeletti S, et al. HSV-TK gene transfer into donor lymphocytes for control of allogeneic graft-versus-leukemia. *Science.* 1997;276:1719-1724.